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E2F transcription factors are believed to control cell growth by being downstream targets of G1 cyclins and the retinoblastoma (Rb) tumor suppressor family. Overexpression and deletion studies in tissue culture and *in vivo* indicate that many E2Fs play a critical role in growth regulation. Since G1 cyclins are upregulated or disregulated and Rb is mutated in several cancers, particularly in breast cancer, upregulation of E2F activity is implicated in cancer development. In order to determine if E2F upregulation is involved in mammary tumorigenesis, retroviruses overexpressing E2F4 were infused into rat mammary glands. No palpable mammary tumors have arisen during five months. Since E2F4 did not cause tumors when overexpressed by itself, a retroviral vector that coexpressed E2F4 and an activated form of c-Ha-ras was made. ras is a known initiator of mammary carcinomas. If E2F plays a role in the progression stage of cancer, the contribution of E2F to cancer development should be more apparent when coexpressed with ras. In addition, coexpression of ras with a dominant negative form of E2F (an E2F with a mutated DNA binding domain) will determine whether ras initiated carcinogenesis is dependent on an E2F pathway.

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Peggy Farnham 8/7/97
PI Signature Date

Traci Lee

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Introduction

Tumors arise when cells escape the controls of cell growth and undergo an inappropriate expansion in cell number. The decision for a cell to divide, and thus expand in number, is made in the G1 phase of the cell cycle before cells replicate their DNA in S phase. It is during the G1 to S phase transition that a cell commits to activating genes whose products are required for DNA replication and cell cycle progression. Many factors which control the G1 to S phase transition of the cell cycle are implicated in the formation of cancer. The activation of several G1/S-phase regulated genes is mediated via a family of transcription factors called E2F (1). The abundance and activity of the E2F transcription factors are tightly regulated by positive and negative regulators of the G1 to S phase transition. These regulators are often mutated in breast cancer in such a way that they could upregulate E2F activity (2). The retinoblastoma (Rb) tumor supressor protein, a direct negative regulator of E2F activity, is lost or inactivated in approximately 30% of breast carcinomas (3, 4). In addition, the activity of positive regulators of E2F activity, such as cyclin D1 and cyclin E, is increased in many breast cancers. Cyclin D1 has been found to be increased in 45% of breast tumor biopsies (5) and studies show altered patterns of expression for cyclin E in numerous breast cancer cell lines and primary breast tumors (6, 7, 8, 9).

The mechanism by which Rb and the G1 cyclins (cyclin D1 and cyclin E) are involved in the formation of breast cancer is unknown, but they are all regulators of E2F activity. While E2F family members have been shown to function as oncogenes in standard transformation assays (10, 11, 12, 13, 14), an *in vivo* investigation of the involvement of E2F in breast cancer development has yet to be done. Because rat mammary carcinoma mirrors human breast cancer in many ways, I proposed to overexpress E2F family members in rat mammary glands using a replication defective retrovirus (15). By expressing E2F proteins, either wildtype or mutant variants, alone or in combination with different oncogenic proteins, I can determine the role E2F proteins play in breast cancer development. I hypothesize that the overexpression of E2F transcription factors can increase the proliferative potential of the breast cells and contribute to neoplastic transformation.

Body

Experimental methods / Procedures

Cloning and plasmids used

The pJLR plasmid and a plasmid containing the acitvated c-Ha-ras gene were obtained from the Gould laboratory (16). The IRES (Internal Ribosomal Entry Site) was obtained from W. French Abderson's laboratory (17). The mE2F1 cDNA was available in our laboraory and the hE2F4 cDNA was obtained from Rene Benards (13, 18). Cloning was done using standard procedures (19).

Cell Culture

Psi- Cre and PA317 cell lines were received from the Gould laboratory and grown in DMEM (Dulbecco's Minimum Essential Media) with 10% FCS (Fetal Calf Serum) and 5% Penicillin / Streptomycin in a 5% CO_2 incubator at 37°C.

Establishment of producer cell lines, production and concentration of retrovirus, and infusion of retrovirus into the rat mammary gland

The procedure for the establishment of producer cell lines, production and concentration of retrovirus, and infusion of retrovirus into the rat mammary gland is described in Wang et al. (16).

Whole cell protein extraction

Cells were grown in T225 flasks and harvested by trypsinization. Cells were pelleted by centrifugation at 1000 rpm for 5 min. in a tabletop centrifuge and the supernatent was removed. Cell pellets were stored at -20° C. Whole cell protein extractions were done by thawing the cell pellet that was harvested from one T225 flask briefly at 37° C. The cells were then kept on ice and 0.5 ml of lysis buffer [50mM Tris, pH 8.0; 5mM EDTA; 150mM NaCl; 0.5% NP-40; 1mM PMSF] was mixed with the pellet. After leaving the cells on ice for 20 min., the cells were further mixed for 1 min. and then the lysate was placed in a 1.5 ml microcentrifuge tube and spun at 14,000 rpm for 5 min. at 4° C in a microcentrifuge. The supernatent is then assayed for protein content and kept for Western studies.

Nuclear protein extraction

Cells were grown in T225 flasks and harvested by trypsinization. The cells were pelleted by centrifugation at 1000 rpm for 5 min. in a tabletop centrifuge and the supernatent as removed. Cells were rinsed with 1X Phosphate buffered saline (PBS). Pellet was resuspended in approximately four times the pellet volume of Dignam-Roeder buffer A (20). After the mixture is allowed to sit on ice for 20 min., a 20-30 second centrifugation in a microgentrifuge at 14,000 rpm is conduted to pellet the nuclei. The

supernatent is removed and the pellet of nuclei is resuspended in Dignam-Roeder Buffer C at halp the buffer A volume used. The nuclear mixture is allowed to sit on ice for 30-35 min., then a 4° C centrifugation in a microcentrifuge at 14,000 rpm for 8-10 min. pellets the insoluble material. The nuclear supernatant is assayed for protein content and kept for Western analysis.

Western analysis

Protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 10% gel by standard techniques (19). Proteins were transferred to nitrocellulose electrophoretically and probed with antibodies against E2F proteins (antibodies from Santa Cruz) or with antibodies against TBP as a standardization control (from Richard Burgess's laboratory) (21). Proteins were detected by enhanced chemiluminescence (reagents and protocol from Amersham).

Results and Discussion:

Initially, I attempted to overexpress E2F1 or E2F4 alone in the rat mammary gland using a procedure developed by Michael Gould. This procedure involves making replication defective retroviruses which express your gene of interest and using the retroviruses to infect rat mammary glands. The Gould laboratory has previously used this system to investigate the affect of Harvey (Ha) ras on rat mammary carcinoma in both a mammary carcinoma-susceptible rat strain, Wistar-Furth (W-F), and a resistant strain, Copenhagen (COP) (16, 22).

The Gould laboratory supplied me with a recombinant retroviral expression vector called pJLR. This Moloney Murine Leukemia Virus (MMLV) -based vector is replication defective and is designed to not allow recombination of the vector into a replication competent form. I placed the mouse E2F1 or human E2F4 cDNA under the control of the MMLV LTR promoter. There is also a SV40 promoter driven neomycin resistance gene in the vector which allows for G418 drug selection of cells which contain this vector.

Two packaging cell lines with different tropisms, supplied by the Gould laboratory, were used to create high titers of the viral vector which is required for the successful infection of the mammary cells. The pJLR vector was first introduced into the ecotropic cell line, psi-CRE, which is only able to infect their natural hosts, mice and rats. The virus produced from these cells were then used to infect the amphotropic cell line, PA317, which infects both its natural hosts and other species, including humans. It has been shown that the use of two packaging cell lines creates higher titers of virus than the use of only one. In addition, the amphotropic retroviral vector has a higher infection efficiency in the rat mammary cells *in situ* than the ecotropic virus. While these replication defective

retroviruses could theoretically infect human cells, a BSL3 (BioSafety Level 3) containment is not necessary. Regardless, I have conducted the E2F1 and E2F4 virus production studies in the McArdle BSL3 facility. Future virus production, however, will be conducted in a BSL2 facility using some BSL3 precautions.

Once I established stably infected PA317 clones through G418 selection, I tested the packaging lines for production of virus and overexpression of the E2F protein. In order to find a PA317 cell line that both produced high virus titers and overexpressed E2F protein, I selected 25 E2F1 packaging clones and 30 E2F4 packaging clones. Of the E2F1 packaging lines selected, some clones produced high virus titers but none of the virus producing clones overexpressed E2F1 protein when assayed by Western analysis. With the E2F1 retrovirus, I infected NIH 3T3 cells (which do not contain retrovirus packaging materials) in order to determine if virus production interfered with protein production. I selected for stably infected NIH 3T3 cell pools with G418 and assayed the cells for E2F1 overexpression by Western analysis. E2F1 overexpression was not detected. I could not get good virus production and overexpression of E2F1 in packaging cell lines. E2F1 is known to cause apoptosis under various conditions when it is overexpressed. It is possible that the packaging cell line selected against the overexpression of E2F1 in order to produce virus. Since I was unable to isolate a packaging clone that both produced E2F1 virus and overexpressed E2F1 protein, I decided to focus my efforts on the E2F4 studies.

While there also seemed to be some selection against the overexpression of E2F4 for virus production, I did find clones which produced virus and overexpressed E2F4 from the 30 E2F4 retrovirus packaging lines I selected. As expected, NIH 3T3 cells infected with the retrovirus overexpressed E2F4 but did not produce virus . E2F4 packaging clone #17 was selected to use for virus collection because it overexpressed the E2F4 and produced virus at a concentration of 4×10^4 viruses/ml. I concentrated the virus supernatent to 5×10^6 viruses /ml using a sucrose cushion and, in a collaboration with the Gould laboratory, a specialist directly infused the concentrated retroviruses into rat mammary glands. In order to detect any oncogenic effects of E2F4, I chose the mammary carcinoma susceptible W-F rat strain for E2F4 infusion. Since activated ras has been shown to cause mammary cancers via this method, I used retroviruses which express an activated form of cellular Harvey ras as my positive control and I chose B-galactosidase (Bgal) expressing retroviruses which do not cause mammary tumors as my negative control.

It has been found that the proliferation status of the rat mammary epithelial cells can affect the infection efficiency of the retrovirus. Perphenizine, a potent mammary mitogen, can indirectly increase the circulating levels of prolactin, thereby increasing mammary DNA synthesis 7-fold and infection rate about 10-fold. Therefore, I treated all rats with three daily subcutaneous injections of perphenizine (3 mg/kg) two days

before virus infusion. Immediately before infusion, I mixed the concentrated virus stocks with polybrene (to facilitate infection) and indigo carmine (as a tracking dye). Female rats, at 50-60 days of age, were etherized and the central duct of each of the 6 lower glands were cannulated with a 27 gauge blunt-ended needle, at which time, 15 µl of virus suspension was infused into the luminal spaces of the gland through the central duct. The virus can then infect the epithelial lining of the duct. 16 W-F rats received the E2F4 virus, four rats received the positive control (activated c-Ha-ras) virus, and four rats received the negative control (Bgal) retrovirus.

One month after virus infusion, I started monitering the rats weekly for the development of palpable mammary tumors. In published results from the Gould laboratory, such tumors arose quickly (5 weeks) after infusion of v-Ha-ras expressing retroviruses and animals were sacrificed 2 weeks later to allow for precise tumor identification and size measurement. In W-F rats, v-Ha-ras gave rise to 0.36 tumors per gland with an average size of 33.4 mm^2 (16). Consistent with these studies, my positive control rats which were infused with an activated form of c-Ha-ras gave rise to very small palpable tumors one month after infusion. Two months after infusion, tumors in some of the positive control rats reached approximately 1cm in diameter and were resected in compliance with the approved animal protocol for this experiment. Histology on the resected tumors identified the tumors as mammary adenocarcinomas. Since I expected that E2F4 would have less dramatic effects on tumor formation, I am monitoring these rats for six months for the appearance of tumors. To date (five months after infusion), no palpable tumors in the E2F4 infused rats and the negative control (Bgal infused) rats have been detected. Four months after infusion two E2F4 infused rats and one Bgal infused rat were sacrificed and their mammary glands were removed and mounted on a slide for histology. No cellular abnormalities were detected in the mammary glands.

At six months after infusion, I will sacrifce the rats and conduct RT-PCR with retrovirus specific primers to determine if E2F4 is still being expressed in mammary cells. I will also conduct Bgal stainings of mammary whole mounts from the Bgal rats to see if I can detect Bgal staining in the gland after six months. This procedure will give an estimate of how many cells in the mammary gland are still producing retroviral protein after six months. Studies done in the Gould laboratory demonstrate that Bgal from this virus can still be detected in the mammary gland whole mounts 18 months after infusion.

Future work / Recommendations

Since my initial studies to date indicate that wildtype E2F4 expression alone in the rat mammary gland does not lead to the formation of mammary tumors, I will look at an E2F4 mutant that has more oncogenic potential in standard transformation assays than the wildtype protein (14).

This E2F4 mutant protein contains a deletion of the pocket binding domain such that it can no longer bind to Rb and its related proteins, p107 and p130. Therefore, these important negative regulators are unable to control the activity of E2F4. Loss or inactivation of Rb, which is seen in approximately 30% of human breast cancers, is believed to play an important role in breast tumorigenesis. Rb can control many transcription factors but the E2F family are the only traanscription factors found to date which link control of the cell cycle with Rb. This study will determine if loss of E2F control is important to the role Rb inactivation plays in breast tumorigenesis.

I have also decided to coexpress E2F with ras. Studies in tissue culture indicate that ras and E2F cooperate to transform rat embryo fibroblasts even though ras or E2F is unable to transform the cells when expressed alone (11, 13). I will express E2F4 with ras. Although I have found that E2F4 does not cause tumors when expressed alone in the rat mammary gland, coexpression of E2F4 with ras may increase the number or size of tumors that arise from ras expression alone. If that is the case, E2F4 can contribute to ras initiated tumerigenesis. A recent study has shown that transgenic coexpression of E2F and ras in mouse skin leads to a severe cancer phenotype which is not seen when ras or E2F is expressed alone (23). While cooperation between ras and E2F has been demonstrated in vivo, results seen in skin can be very different than what is seen in the mammary gland. These studies may give insight into the tissue specific environment of the mammary gland.

I will also coexpress ras with a dominant negative form of E2F (with a mutated DNA binding domain) which will interfere with the ability of all E2F transcription factors to bind DNA. By doing this, I can determine if ras causes tumors using a E2F dependent pathway in the mammary gland. Ras has been shown to activate many different pathways (24). One of the proteins that ras activates is cyclin D1 which can increase E2F activity (25, 26). If dominant negative E2F expression causes a decrease in the number and/or size of ras initiated tumorigenesis, ras must work through an E2F pathway to cause cancer.

In order to coexpress ras and E2F, I will express the two proteins from the same MMLV LTR promoter. E2F is the first gene driven by the promoter and the activated form of c-Ha-ras is the second gene. There is an Internal Ribosmal Entry Site (IRES) between these two genes which allows the ras gene to be recognized by the translation machiney for protein expression. I received the IRES from W. French Anderson (17). Because ras expression is dependent on the IRES, ras will only be expressed at approximately 20% the expression levels of E2F. Therefore, the tumorigenic effects of ras are likely to be diminished compared to the other ras studies which had higher expression levels. In order to determine the tumorigenic potential of this new ras retrovirus, I have made a vector that expresses the luciferase gene directly downstream of the LTR and expresses ras from the IRES. The LTR-luciferase-IRES-ras vector should have similar levels of ras expression as the LTR-E2F-IRES-ras vector.

In addition to the above alterations, I will be using a different retroviral vector that expresses an enchanced form of the green flourescent protein (eGFP) instead of the neomycin resistance gene as a selectable marker. eGFP is a protein marker that will emit a green light in the presence of blue light. Therefore, cells which contain the eGFP protein can can be visualized in a microscope or be selected by flow cytometry analysis and cell sorting without perturbing the cell in any way. This powerful tool will allow for quicker titering of single cell clones and visualization and selection of infected cells *in vitro* and *in vivo* (27). I recieved a retroviral vector which contained eGFP from Phil Watson in the Gould laboratory.

Currently, I am almost done with cloning the new retroviral vectors and I will be doing the tissue culture work to make viruses soon.

Conclusions

Overexpression of E2F4 alone does not cause tumors in the Wistar Firth rat mammary gland.

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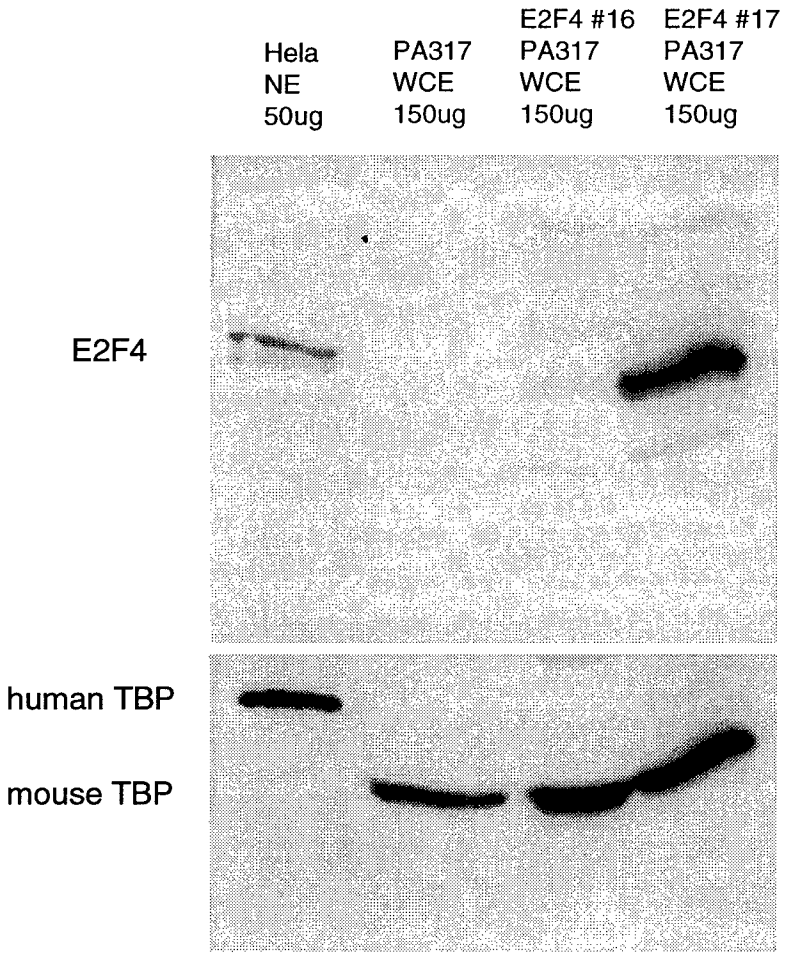
Figure 1

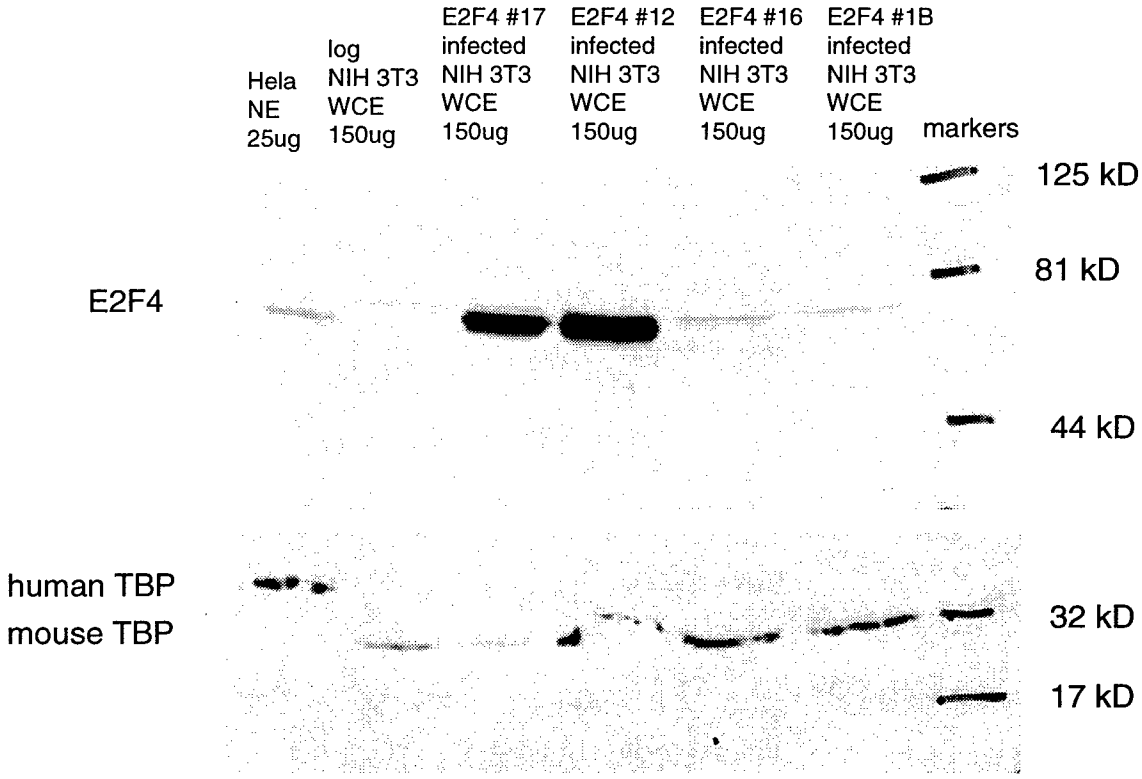
E2F4 is being overexpressed in E2F4 PA317 clone #17



Western of E2F4 PA317 clone #17. Hela NE was used as a positive control for detecting E2F4. WCE from PA317 cells was used to determine the background levels of E2F4 before the E2F4 viral construct was introduced. As a comparison, WCE from a different E2F4 PA317 clone (clone #16) was loaded. After the blot was probed with E2F4 antibodies, the blot was reprobed with TBP (tata binding protein) antibodies as a standardization control. NE= nuclear extract. WCE=whole cell extract.

Figure 2

E2F4 is being overexpressed in NIH 3T3 cells infected with virus from E2F4 PA317 cells clone #17



Western of NIH 3T3 cells stably infected with virus supernatent from E2F4 PA317 clones. Hela NE was used as a positive control for detecting E2F4. WCE from uninfected log growing NIH 3T3 cells was used to determine the background levels of E2F4 before the E2F4 viral construct was introduced. The last lane was loaded with molecular weight markers. After the blot was probed with E2F4 antibodies, the blot was reprobed with TBP (tata binding protein) antibodies as a standardization control. NE= nuclear extract. WCE= whole cell extract.